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TITLE: Investigating the Role of Cooperative Interactions Between
the Neu Proto-oncogene and the Other erbB Family Members in
Rat Mammary Carcinogenesis

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In order to generate a rat model of neu-induced mammary carcinogenesis, we have created transgenic rats that utilize the mouse mammary tumor virus promoter to overexpress the rat neu proto-oncogene in the mammary gland. A nuclease protection assay was used to quantify the relative overexpression of neu message within the mammary gland and rats of both sexes were observed until at least 14 months of age. In the most characterized line (line 6500), transgenic virgin females and males at 12 weeks of age overexpressed neu in the mammary gland. The expression level of neu was equal between the two sexes. Despite this neu overexpression, mammary carcinomas did not develop in any of the transgenic virgin females at 14 months or 18 months. In striking contrast to the females, 77.8% and 100% of the transgenic males developed mammary carcinomas at 14 and 18 months of age, respectively. Sequence analysis from 13 of the mammary carcinomas revealed that the neu transgene had not undergone any spontaneous mutations in the transmembrane domain and adjacent extracellular domain, regions which have been reported to be mutated in neu transgenic mice. To assess the role of hormones in the observed mammary carcinogenesis, both male and females were gonadectomized at 8 weeks of age and sacrificed at 14 months of age. Mammary carcinomas did not develop in any of the castrated transgenic males while 100% of the intact transgenic males developed multiple mammary carcinomas. Transgenic females did not develop mammary carcinomas regardless of gonadal status. These results suggest that in the context of HER2/neu overexpression, androgens may be powerful promoters of mammary carcinogenesis..

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BODY

I. Research Period: July 1, 1996-June 30, 1997

TITLE: Evaluating Gene Therapy Approaches for the Prevention of Breast Cancer

INTRODUCTION

Breast cancer strikes 1 in 8 American women during the course of her lifetime and there is currently little that can be done to prevent the disease. The field of gene therapy could possibly provide a new approach for the prevention of breast cancer. The premise of gene therapy relies upon the introduction of beneficial genes into the patient. This is usually accomplished through the use of replication-defective viruses as a vehicle for conveying the beneficial genes.

Both retroviruses and adenoviruses have been used for this purpose. Each type of virus has strengths and weaknesses. Although retroviruses are limited to infecting replicating cells, they offer the advantage of stable integration of the viral genes into the host's genome. This property of retroviruses makes it theoretically possible to achieve long-term expression of a beneficial gene. Adenoviruses, on the other hand, can infect cells regardless of cellular proliferation. However, adenoviruses are not capable of stable long-term infection for two major reasons. First, the adenovirus genome remains in the cell for only a short time as an extranuclear plasmid. Secondly, adenovirus infection tends to elicit an aggressive immune response due to the expression of viral antigens on the infected cell.

These differential properties of retroviruses and adenoviruses could be exploited for alternative gene therapy approaches for the prevention of breast cancer. In a cytostatic approach, genes would be conveyed to mammary epithelial cells by retroviral vectors. These vectors would contain genes that are negative regulators of cell growth and could potentially be expressed long-term from the stably integrated provirus. The net result would be permanent growth inhibition of the infected mammary epithelial cells. Adenoviruses could be used in a cytotoxic gene therapy approach. These adenoviral vectors would transfer toxic genes to the mammary epithelium and, additionally, the host immune response would be recruited against the infected cells. If nearly 100% of the mammary epithelial cells could be infected, this approach could result in the selective ablation of the epithelial population. The result would be removal of the cellular origin of mammary carcinomas.

During this research period, we evaluated the efficacy of gene therapy approaches for the prevention of mammary carcinogenesis in the rat using both retroviral and adenoviral vectors. Additionally, we also evaluated the cytostatic gene therapy approach for the prevention of mammary carcinomas following carcinogen challenge.

MAJOR RESEACH ACCOMPLISHMENTS

Relative Infection Efficiencies of Adenoviral and Retroviral Vectors *in situ*

Adenoviral or retroviral vectors containing the lacZ reporter gene were infused into the mammary glands of rats. At different time points following infusion, the infused mammary glands were stained *in situ* with X-gal to determine the relative number of infected cells, which turn blue following X-gal staining. Adenovirus infected the mammary epithelial cells to a much larger degree compared to retrovirus. Both types of viruses infected end bud cells. The primary and secondary ductal cells were not infected by either virus.

Evaluation of a Cytostatic Gene Therapy Approach for the Prevention of Mammary Carcinogenesis

Transforming growth factor β -1 (TGF β -1) is a known negative growth regulator of some epithelial cells. To determine if TGF β -1 could prevent mammary carcinogenesis, rats were treated with the mammary carcinogen N-nitroso-N-methylurea (NMU). Following NMU treatment, the rats were infused with a retroviral vector containing a constitutively active TGF β -1. 19 weeks following NMU treatment, the experiment was terminated. Rats that received the TGF β -1 retrovirus did not gain any reduction in the incidence, multiplicity, or latency of mammary carcinomas compared to rats not treated with TGF β -1.

DISCUSSION

The efficacy of gene therapy approaches for the prevention of breast cancer was evaluated during this research period. Using the rat as an experimental model, it was determined that both adenoviral and retroviral vectors can infect the mammary epithelial cells *in vivo*. Neither viral type was able to infect the cells of the mammary duct. The success of the proposed cytotoxic gene therapy approaches depended on a nearly 100% infection efficiency of the adenoviral vectors. Because the ductal cells are not infected by adenovirus, the proposed cytotoxic gene therapy approaches could not be successful. As an example of a cytostatic gene therapy approach, it was determined if retroviral expression of TGF β -1 could inhibit the development of mammary carcinomas following a challenge with the mammary carcinogen NMU. Treatment with the TGF β -1 retrovirus did not have any beneficial effect in inhibiting NMU-induced mammary carcinogenesis. The initial characterization of the gene therapy approaches done during this period indicated that the proposed experiments would not be successful. All further attempts to develop a gene therapy approach for the prevention of breast cancer were aborted.

II. Research Periods: July 1, 1997-June 30, 1998 and July 1, 1998-June 30, 1999

TITLE: Investigating the Role of Cooperative Interactions Between the Neu Proto-oncogene and the Other erbB Family Members in Rat Mammary Carcinogenesis

INTRODUCTION

The type 1 family of tyrosine kinase growth factor receptors include four closely related members: EGFR, erbB2/HER2/neu, erbB3/HER3, and erbB4/HER4. These proteins play important roles in the growth and differentiation of mammalian cells. Aberrant overexpression of these proteins has also been associated with some human malignancies. In particular, 25% of human breast cancers overexpress neu. Despite this common association, the exact role that neu plays in the etiology of breast cancer remains unclear.

A number of rodent models have been developed in the hope of better understanding the role of neu in breast cancer. Transgenic mice that overexpress either a constitutively active neu mutant or the wild-type proto-oncogene develop mammary carcinomas. In these models, neu becomes activated by mutation which likely results in the formation of neu homodimers. Besides mutational activation, the tyrosine kinase activity of neu can become functional through heterodimerization with other members of the erbB family. After activation of the neu kinase activity, a number of downstream proteins are phosphorylated and cellular growth is stimulated. This can result in an increased transforming potential. For example, dual overexpression of EGFR and neu results in the transformation of NIH 3T3 cells. Although heterodimerization of neu with the other erbB family can clearly promote transformation in *in vitro* systems, the same has not been demonstrated in *in vivo* models. Therefore, during this research period, we asked if dual overexpression of neu with the other erbB family members would enhance mammary carcinogenesis in a transgenic rat model of neu. We chose to develop a transgenic rat model for neu because rat mammary carcinogenesis more closely resembles its human counterpart.

MAJOR RESEARCH ACCOMPLISHMENTS

Generation of Rats Transgenic for the Neu Proto-oncogene

Three lines of transgenic rats were created that contain the rat neu proto-oncogene under the control of the mouse mammary tumor virus promoter (MMTV). These lines were designated 4311, 6490, and 6500. Initial characterization of these three lines appeared to show that aged transgenic females of all three lines do not develop mammary carcinomas. In addition, males of lines 4311 and 6490 are also without any phenotype. However, males of line 6500 develop multiple mammary carcinomas beginning at 10 months of age. By 14 months of age, 78% of the transgenic males were positive for mammary

carcinomas. Preliminary expression analysis at the RNA level indicated that transgenic females of line 6500 overexpressed neu in the mammary gland. Females of the other two lines did not appear to overexpress neu.

Infusion of Retroviruses Expressing the erbB Family Members

This experiment addressed if overexpression of the erbB family members could cooperate with neu in mammary carcinogenesis. Retroviral vectors were constructed that contain the human forms of EGFR, erbB3, or erbB4. Initially, these genes were placed into a retroviral vector construct that utilizes green fluorescent protein (GFP) as the reporter gene. However, a vector containing both GFP and an activating neu mutant had a significantly reduced ability to transform the rat mammary gland *in vivo*. This result was unexpected. Because GFP appeared to somehow interfere with tumorigenesis in the mammary gland, the erbB genes were expressed in a retroviral vector using neomycinphosphotransferase (neo) as the reporter gene. Extensive experience with neo in our laboratory reveals no inhibitory effect of mammary tumorigenesis. These recombinant retroviruses were infused into the mammary glands of 6500 transgenic female rats and their non-transgenic littermates. Six months after infusion, all of the rats were free of mammary carcinomas.

DISCUSSION

In order to develop a rat model of neu-induced tumorigenesis, three lines of transgenic rats were created that express the rat neu proto-oncogene under the control of the MMTV promoter. It was expected that female transgenic rats would develop mammary cancer, as is the case in mice transgenic for the neu proto-oncogene. However, up to this point, female transgenic rats have remained free of mammary carcinomas. Although two of the lines appear to be non-useful due to lack of transgene expression, line 6500 females do overexpress neu in the mammary gland. It is somewhat surprising, then, that females of line 6500 remain cancer free. It was not expected that male transgenic rats would develop any pathology, as male mice transgenic for neu are without any reported phenotype. However, nearly all line 6500 males to date develop multiple mammary carcinomas by 14 months of age.

Because overexpression of neu is not sufficient for mammary carcinogenesis in line 6500 transgenic females, we next addressed if dual overexpression of neu and the other erbB family members would cooperate to enhance mammary carcinogenesis. In order to overexpress the other erbB members, retroviral vectors containing the human forms of EGFR, erbB3, or erbB4 were infused into the mammary glands of line 6500 transgenic females. However, by six months post-infusion, no mammary carcinomas had developed in any of the rats (transgenic and non-transgenic). The negative result of this experiment is difficult to interpret for several reasons. For example, the endogenous ligands, which are necessary for receptor activation, could have been present at insufficient levels to promote carcinogenesis. Alternatively, heterodimerization may be insufficient to induce mammary carcinogenesis. In light of the phenotype of line 6500 transgenic males, androgens may be necessary co-factors for mammary carcinogenesis

induced by the erbB family of growth factor receptors. Whatever the cause for these results, further investigation into the cooperative interactions of neu and the other erbB members in mammary carcinogenesis is not warranted at this time. However, the unexpected finding of mammary carcinomas exclusively in transgenic males does merit further investigation.

III. Research Period: July 1, 1999-June 30, 2000

TITLE: The Role of the Neu Proto-oncogene in the Etiology of Breast Cancer

INTRODUCTION

The neu proto-oncogene is amplified and overexpressed in 25% of human breast cancer. Although neu is clearly associated with breast cancer, the exact role that it plays in the development of the disease has not been definitively established. A number of rodent models have been developed in order to address this issue. Transgenic mice that overexpress either a constitutively active neu mutant (1,2) or the wild-type neu proto-oncogene (3) develop mammary carcinomas. However, in the latter model, the neu proto-oncogene transgene spontaneously mutates to a constitutively active form (4). Hence, in both of these transgenic mice models, the mammary carcinomas result from overexpression of an active neu mutant. Unlike these transgenic mice models, human breast tumors are associated exclusively with overexpression of the non-mutated neu proto-oncogene (5). Therefore, in order to fully model the human disease, it is necessary that mammary tumors in rodent models are derived predominantly from overexpression of the neu proto-oncogene.

Besides the mouse, the rat is also used in breast cancer research. Rat mammary cancer mimics some of the characteristics of the human disease, namely a high percentage of ER+ carcinomas. For this reason, we chose to create transgenic rats that overexpress the neu proto-oncogene in the hope of developing an alternative rodent model for neu-induced carcinogenesis. As previously reported, we have created one such transgenic line (6500) in which mammary carcinomas arise exclusively in the male. This observation raises the possibility that androgens and neu cooperate in mammary carcinogenesis.

Epidemiological studies have consistently shown an association between elevated levels of testosterone and breast cancer in post-menopausal women (6). The fact that a high percentage of breast tumors express the androgen receptor (7) lends support to the idea that direct testosterone signaling may play a causative role in breast cancer. The transgenic model that we report here may help to reveal the roles that androgens and neu play in the etiology of breast cancer.

MAJOR RESEARCH ACCOMPLISHMENTS

Confirmation of the Phenotype of Line 6500 MMTV-neu N Transgenic Rats

Previously we reported preliminary findings on the phenotype of rats transgenic for the neu proto-oncogene. During this research period, we have extended these observations on a larger number of rats and this phenotype has remained the same. As summarized in Table 1, 78% of transgenic males developed multiple mammary carcinomas by 14 months of age. Non-transgenic males were completely tumor free. Likewise, both transgenic and non-transgenic females were free of mammary carcinomas at 14 months of age.

Both Transgenic Males and Females Overexpress Neu to an Equal Degree in the Mammary Gland

The relative expression levels of neu RNA in the transgenic mammary gland were quantified using a nuclease protection assay. As a control, the levels of neu were also determined in non-transgenic littermates. At 12 weeks of age, both male and female transgenic rats overexpressed neu to a comparable level in the mammary gland. This data is summarized in Table 2.

The Neu Transgene is not Mutated in the Male Mammary Carcinomas

Male mammary carcinomas were micro-dissected using a Laser Capture Microscope. DNA was isolated from dissected carcinoma tissue and the neu transgene was subjected to PCR amplification in order to amplify an area of neu spanning the transmembrane and the adjacent extracellular domains. This region of neu has previously been shown to be frequently mutated in mammary carcinomas arising in the neu transgenic mice (4). The PCR fragment was completely sequenced from 13 mammary carcinomas and found to not contain any mutations.

Retroviral Expression of Neu with Mutations in the Juxtamembrane Domain is Sufficient to Induce Mammary Carcinogenesis in Female Rats

In the neu transgenic mice, an area of the extracellular domain adjacent to the transmembrane domain is frequently deleted, resulting in an activated and powerfully transforming neu. The cDNAs for two of these neu mutants were kindly provided by Dr. William Muller and were subcloned into a retroviral expression vector. Infusion of these constructs into the mammary glands of female rats readily induced an extremely large number of mammary carcinomas in 100% of the treated rats. Due to the tumor burden, these rats were sacrificed 7 weeks post-infusion.

Castration Inhibits the Development of Male Mammary Carcinomas

At 8 weeks of age, both transgenic males and females underwent a bilateral gonadectomy. Non-transgenic littermates were similarly treated and served as negative controls. These rats were sacrificed at 14 months of age. In the transgenic male, castration completely inhibited the development of mammary carcinomas. In contrast, 100% of the intact transgenic males had numerous mammary carcinomas. As seen before, intact transgenic females did not develop mammary carcinomas and ovariectomized females were likewise free of mammary cancer. This data is summarized in Table 3.

DISCUSSION

We have previously reported on the generation of one line of transgenic rats that overexpress the neu proto-oncogene in the mammary gland. Initial indications were that these rats develop mammary carcinomas only in the males. Here, we extended these observations to a larger number of female rats and this sex-specific phenotype has been confirmed.

This unexpected phenotype is in complete contrast to the phenotype of mice transgenic for the neu proto-oncogene. Those mice develop mammary cancer only in the females and males are without any reported pathology. However, these carcinomas arise from mutational activation of the neu transgene. The result is the conversion of the original neu proto-oncogene to a potent oncogene. Because this spontaneous mutation of the neu transgene is such a common event in the transgenic mice, the possibility exists that the same phenomenon could be driving mammary carcinogenesis in the transgenic rats. To address this issue, mammary carcinomas from transgenic males were subjected to micro-dissection using a Laser Capture Microscope. This procedure was done to ensure that only epithelial cells would be analyzed. An area of the neu transgene spanning the transmembrane and adjacent extracellular domains was PCR amplified from dissected carcinoma tissue. This is the region of neu that undergoes spontaneous mutational activation in the neu transgenic mice. Sequence analysis of the neu transgene from 13 male mammary carcinomas failed to reveal any mutations in this critical area. Due to technical limitations, we cannot absolutely rule out the presence of activating mutations in a small percentage of the total pool of transgenic neu copies. However, if these mutations are still occurring at a very low frequency, they either are not occurring in female rats or are insufficient to induce mammary carcinogenesis in the females. To address the latter point, we infused retroviral constructs containing activated neu mutants that were originally isolated from the neu transgenic mice into the mammary gland of female rats. These constructs readily transformed the female rat mammary gland, indicating that these neu mutations are sufficient for mammary carcinogenesis in the female rat. We therefore believe that spontaneous activating mutations of the neu transgene do not occur in the neu transgenic female rat. These mutations could still be occurring in the neu transgenic male rats at a frequency below the detection threshold. However, we do not know of any examples where proto-oncogenes undergo mutational

activation selectively in male rodents. Collectively, our data argues against the occurrence of spontaneous mutations of neu in the transgenic rat. Rather, it seems to be the case that mammary carcinomas in the neu transgenic rat are associated with overexpression of the non-mutated neu proto-oncogene. This represents an important species difference between the mouse and rat in neu mediated mammary carcinogenesis. This issue is significant because in humans, mutational activation of neu has not been reported. Rather, human breast tumors are associated with amplification and overexpression of the neu proto-oncogene. Therefore, in order to completely model the role of neu in the human disease, it becomes important to consider the mutational status of neu within the rodent mammary carcinomas.

Since we do not believe that mutational activation of neu is responsible for the observed sex-specific mammary carcinogenesis in the transgenic rat, we next addressed other mechanisms that could account for this unique phenotype. The MMTV promoter used to drive expression of neu in both the transgenic rat and mouse is known to be responsive to steroid hormone regulation. It is therefore conceivable that transgenic male rats express considerably higher levels of neu compared to transgenic female rats due to differential regulation of the MMTV promoter. To quantify the relative degree of neu RNA overexpression, a nuclease protection assay was used using an oligonucleotide probe directed against neu. The relative levels of neu in the mammary gland were determined for both transgenic males and females. Non-transgenic littermates served as negative controls to establish the baseline level of neu expression in the mammary gland. The results showed that the neu RNA levels did not differ substantially in the two sexes. Therefore, the sex-specific phenotype cannot be explained by differential neu expression levels in males vs. females.

The observed phenotype of our transgenic rats strongly suggests that the sex hormones are playing a highly significant role in the induction of mammary carcinogenesis. Assuming this to be the case, we wanted to next determine if the male hormones are necessary for mammary carcinogenesis. Alternatively, the female hormones may be inhibiting mammary carcinogenesis in the transgenic female. To determine which of these two situations may be occurring, both transgenic males and females were gonadectomized at 8 weeks of age and were sacrificed at 14 months of age to determine the incidence of mammary cancer. Transgenic females, whether intact or ovariectomized, did not develop mammary cancer indicating that the ovarian hormones were not inhibiting mammary carcinogenesis in the transgenic female. As seen before, 100% of the intact transgenic males had developed multiple mammary carcinomas by 14 months of age. In striking contrast, castration completely inhibited mammary cancer in the transgenic male. Therefore, mammary carcinogenesis in the neu transgenic male rat is completely dependent upon a male hormonal environment.

KEY RESEARCH ACCOMPLISHMENTS

- Transgenic rats were created that overexpress the neu proto-oncogene in the mammary gland under the regulation of the MMTV promoter.
- Both male and female transgenic rats equally overexpress neu in the mammary gland.
- Mammary carcinomas arise exclusively in the transgenic male beginning at 10 months of age.
- The neu transgene from the male mammary carcinomas is not mutated in the transmembrane and adjacent extracellular domains.
- Carcinogenesis in the neu transgenic male is completely dependent on an intact hormonal environment.

REPORTABLE OUTCOMES

Abstracts and Presentations

1. 91st Annual Meeting of the American Association for Cancer Research, April 2000, San Francisco, CA
2. Era of Hope Department of Defense Breast Cancer Meeting, June 2000, Atlanta, GA

Funding Applied For

1. Supplemental grant from the National Cancer Institute (received)
2. Idea grant from the Department of Defense (applied for June, 2000)

CONCLUSIONS

During the period of this reward, the major accomplishment was the generation of a line of transgenic rats that overexpress the neu proto-oncogene in the mammary gland. These rats unexpectedly develop mammary cancer exclusively in the male and this carcinogenesis is completely dependent on the male hormonal environment. This is in contrast to transgenic mice that contain the same transgenic construct. Those mice develop mammary cancer only in the females. However, the carcinomas that arise in the transgenic mice are associated with an activated neu mutation which arises spontaneously during carcinogenesis. Unlike the transgenic mice, mammary carcinomas in our transgenic rats are from overexpression of the wild-type neu proto-oncogene. This mirrors the situation in humans, where mutations in neu have not been reported. Therefore, our transgenic rat model may represent the first transgenic rodent model of neu where carcinomas are induced predominantly by the proto-oncogene.

Our transgenic rat model may be directly relevant to human breast cancer. First of all, it provides a model for male breast cancer. Although male breast cancer is a very rare disease, it is still a serious medical condition for those diagnosed with it. Additionally, there is little if any basic research conducted in male breast cancer which contributes to a poor understanding of this disease. We are not aware of any adequate models for male breast cancer and our neu transgenic model may provide the first such relevant model for this deadly disease.

Besides being directly relevant to male breast cancer, our transgenic model may reveal new insights into the role of neu in the etiology of female post-menopausal breast cancer. It has been well established that some post-menopausal women have abnormal levels of testosterone and this correlates with breast cancer. It has not been determined if testosterone is playing a direct or an indirect role in these cases. Because testosterone can be converted to estradiol, the positive association seen between testosterone levels and breast cancer may simply be due to the stimulation of the breast cells by estradiol via testosterone. However, this may be an overly simplistic view. Most breast cancers express the androgen receptor so there is certainly reason to believe that abnormal levels of testosterone in post-menopausal women are directly beneficial to the growth of the cancer. In addition, a high androgen environment and neu overexpression may be synergistic in inducing mammary carcinogenesis. Indeed, it has been recently shown that neu and the androgen receptor are functionally linked in prostate cancer cell lines (8). Our transgenic rat model may, therefore, reveal that the ability of the neu proto-oncogene to induce mammary carcinogenesis is dependent on the androgen receptor pathway.

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MAMMARY CARCINOMAS DEVELOP ONLY IN TRANSGENIC MALES

GROUP	AGE AT NECROPSY	PERCENTAGE OF RATS WITH CARCINOMAS
NEU/+ MALE	14 months	78 (7/9)
	18 months	100 (3/3)
+/+ MALE	14 months	0 (0/11)
NEU/+ FEMALE	14 months	0 (0/11)
	18 months	0 (0/4)
+/+ FEMALE	14 months	0 (0/9)
	18 months	0 (0/6)

Mean # Carcinomas/Rat: 8.5
Range: 0-35 Carcinomas/Rat

**TABLE 1: Rats were sacrificed at 14 or 18 months of age
and the number of mammary carcinomas/rat was determined.**

MALE AND FEMALE TRANSGENICS EQUALLY OVEREXPRESS NEU IN THE MAMMARY GLAND

	GENOTYPE	RELATIVE NEU EXPRESSION
MALE	NEU/+	3.4
	+/+	1.0
FEMALE	NEU/+	2.6
	+/+	1.0

TABLE 2: Total RNA was isolated from mammary gland samples and the level of neu expression was determined by a nuclease protection assay. Rat neu was used as a probe. The levels of neu RNA were normalized to 28S rRNA.

CASTRATION INHIBITS THE DEVELOPMENT OF MAMMARY CARCINOMAS IN THE TRANSGENIC MALE

GROUP	TREATMENT	PERCENTAGE OF RATS WITH CARCINOMAS
NEU/+ MALE	NONE	100 (7/7)
	CASTRATION	0 (0/7)
+/ MALE	NONE	0 (0/6)
	CASTRATION	0 (0/7)
NEU/+ FEMALE	NONE	0 (0/6)
	OVARIECTOMY	0 (0/6)
+/ FEMALE	NONE	0 (0/8)
	OVARIECTOMY	0 (0/8)

TABLE 3: Male and female rats (neu/+ and +/+) underwent gonadectomy or a sham procedure at 56 days of age. The rats were sacrificed between 419-430 days of age.

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Personnel Receiving Pay

Philip Watson